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The Role of Carbohydrates on the Salt Tolerance of Jojoba [*Simmondsia chinensis* (Link)] Explants *in Vitro*

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Summary

Jojoba [*Simmondsia chinensis* (Link) Schneider.] explants were cultured *in vitro* under four levels of sodium chloride concentration during the shoot proliferation stage. The dry weight of explants as well as their water and carbohydrate content were determined during a three-month period at month intervals. Jojoba explants were tolerant to salinity up to a certain level (0.66 % w/v sodium chloride), showing increased water uptake and dry weight compared to the control. Sucrose and pinitol content were found to be higher in explants grown under saline conditions, while the opposite stood for glucose. Fructose, inositol and starch content did not exhibit any significant changes with salinity. Carbohydrates may play an osmoprotective role in jojoba salt tolerance under *in vitro* conditions, by enhancing water uptake from the medium up to a certain salinity level (0.66 % w/v), revealing thus an adaptive response of the species against the increased salinization of the growth medium.

Zusammenfassung

Die Wirkung von Kohlenhydraten auf die Salztoleranz von Jojoba-Explantaten [*Simmondsia chinensis* (Link)] *in vitro*. Jojoba-[*Simmondsia chinensis* (Link) Schneider.] Explantate wurden während der *in vitro* Proliferationsphase bei vier verschiedenen Natriumchlorid-Konzentrationen kultiviert. Das Trockengewicht der Explantate sowie ihr Wasser- und Kohlenhydratgehalt wurden während einer dreimonatigen Periode in monatlichen Abständen ermittelt. Jojoba-Explantate waren bis zu einer bestimmten Salzkonzentration (0.66 % w/v Natriumchlorid), tolerant und reagierten im Vergleich zur Kontrolle mit Erhöhung von Wasseraufnahme und Trockengewicht. Der Saccharose- und der Pinitolgehalt waren in den Explantaten, die unter Salzeinfluss kultiviert wurden, höher, während das Entgegengesetzte für Glukose zutraf. Fructose-, Inositol- und Stärkegehalt zeigten keine signifikanten Änderungen mit dem Salzgehalt. Bis zu einer Salzkonzentration von 0.66 % w/v können Kohlenhydrate durch ihre Steigerung der Wasseraufnahme aus dem Substrat eine osmoprotektive Rolle in der Jojoba-Salztoleranz unter *in vitro* Bedingungen spielen und folglich eine Anpassung an erhöhte Salzkonzentrationen im Substrat aufdecken.

Key words. hexose – osmoregulation – relative growth rate – sodium chloride – starch-tolerance index.

Introduction

Salinity and drought stress reduce growth and plant productivity more than any other environmental factor (BOHNERT and SHEN 1999). Most plants increase their cellular concentration of osmotically active compounds, termed compatible solutes, when in danger of becoming desiccated by either drought or external lowering of the osmotic potential, due to increases of soil salinity (FORD 1984)

Salt stress affects many physiological and biochemical processes in plants. Amongst the major effects on plant metabolic pathways are those involving carbohydrate metabolism, with the accumulation of sugars and a number of other organic solutes (ASHRAF et al. 2002; KAUR et al. 2003). Carbohydrates are frequently associated with active osmotic adjustment (DUBEY and SINGH 1999; ASHRAF et al. 2002; MELONI et al. 2003) and have long been known to increase in a wide range of plants grown under saline conditions (PEREZ-ALFOCEA and LARHER 1995; TATTINI et al. 1996; GUCCI et al. 1998; BOHNERT and SHEN 1999; KAFI et al. 2003).

Jojoba [(*Simmondsia chinensis* (Link) Schneider.] is considered to be a relatively salt tolerant plant species (BENZIONI et al. 1992; MILLS et al. 1997). Nevertheless, salt damage can occur and differences in response among clones have been observed (BENZIONI et al. 1992; BOTTI et al. 1998) indicating the possibility of breeding cultivars with increased salt resistance or tolerance. According to BENZIONI et al. (1992) and MILLS and BENZIONI (1992) *in vitro* nodal segments of jojoba respond to salinity in a similar way as the whole plant. In order to be able to evaluate clones at a relatively early stage of growth, *in vitro* culture-selection can become a powerful tool based on some biochemical characteristics, which can be used in the early selection for tolerance.

In the present study, the possible contribution of carbohydrates to salinity tolerance is investigated, so as to determine whether they may be used as a research tool in the selection of salt tolerant jojoba clones.

Materials and Methods

In vitro *culture and salt treatments*

Jojoba explants originated from an one year culture, maintained by successive subcultures in a basal medium described by ROUSSOS et al. (1999), supplemented with 4 mg l^{-1} BA for invigoration. At least thirty single node and shoot tip explants were used per treatment. Salinity treatments (sodium chloride 0 % w/v = control, 0.33 % w/v = low, 0.66 % w/v = medium and 0.99 % w/v = high salinity level) were imposed as described earlier (ROUSSOS and PONTIKIS 2003). Explants remained at shoot proliferation stage under saline conditions for three months while their growth parameters were monitored as has been already reported by ROUSSOS and PONTIKIS (2003). At the end of every month, ten flasks from each treatment were withdrawn in groups of five (two groups per treatment) and lyophilised.

The water content of explants (expressed as moisture per gram dry weight) was recorded along with the relative growth rate (R*GR*, g $month^{-1}$), in order to evaluate jojoba growth under salt stress conditions. R*GR* was calculated based on the increases in dry weight over the cultivation period, using the formula for exponential growth (MAKELA et al. 2003):

$$RGR = \frac{In(\text{final dry weight}) - In(\text{initial dry weight})}{\text{Time}}$$

Furthermore, a tolerance index (TI) was used to evaluate jojoba tolerance to salinity stress, using the following formula (DUTTA GUPTA et al. 1995):

$$TI = \frac{\text{Dry wt. on NaCl medium}}{\text{Dry wt. on NaCl free medium}} \times 100$$

Soluble sugars and starch extraction and analysis

Soluble sugars and starch were extracted from approximately 30 mg dry weight of explants, according to the method of ROCCA and ROUCHOUSE (1976) with some modifications (VEMMOS 1999). Soluble sugars samples were diluted into 3 ml of water HPLC grade (BDH), filtered through a nylon syringe filter (0.45 μm) and analysed using HPLC (Hewlett Packard HP 1050). Analysis was performed with a Hamilton HC-75 cation exchange column, calcium form (Ca^{2+}), at 65 °C with water as eluent at a flow rate of 0.8 ml min^{-1}. Detection of soluble sugars and polyols was accomplished by the use of a refractive index detector (HP 1047 A) and each sample was analysed twice. Starch concentration was determined colorimetrically according to the method of DEKKER and RICHARDS (1971) with some modifications (VEMMOS 1999).

Statistical analysis

Experiments with jojoba explants were arranged in a completely randomised design with thirty explants per treatment and two replications. The data from the biochemical analyses were analyzed as a two-way Anova, with one factor being time and the other the salt treatment. For the figures, SEs were calculated from the residual variances. Statistical analysis was performed using Statgraphic plus, version 2.1 for Windows.

Results

Tolerance index and relative growth rate of explants

Jojoba explants exhibited high tolerance indexes up to the medium level (0.66 % w/v) of salt concentration. At the high level of salinity (0.99 % w/v), explants exhibited the lowest tolerance index, reaching 50 % over the control (Table 1). When jojoba explant growth was examined in terms of relative growth rate, explants continued to grow at a similar rate to that of the saline-free control explants, up to the medium salinity level (Table 1). Furthermore, the relative growth rate of explants grown under saline conditions exhibited a drastic decline during the third month, while explants grown under control conditions continued growing with similar growth rates.

Carbohydrate content of explants

The HPLC carbohydrate profile of jojoba explants revealed some major carbohydrates such as sucrose, glucose and fructose as well as the cyclitol pinitol and its precursor inositol (DITTRICH and KORAK 1984). D-ononitol and D-chiro-inositol were not detected in jojoba explants.

The concentration of sucrose, glucose, pinitol and total hexoses (fructose+glucose) were significantly affected by salt concentration (Table 2). The sucrolysis

Table 1. Tolerance index and growth rates of jojoba explants as affected by salinity during the shoot proliferation stage (Mean ± standard error).

Salinity treatments	Tolerance index during the 3rd month (%)	Growth rate (g $month^{-1}$)	
		1st to 2nd month	2nd to 3rd month
Control	–	0.96 ± 0.01	0.95 ± 0.01
Low	110 ± 8	1.06 ± 0.04	0.74 ± 0.02
Medium	125 ± 11	1.06 ± 0.02	0.84 ± 0.04
High	50 ± 4	0.76 ± 0.01	0.38 ± 0.02

Table 2. Probabilities of effects of salinity level and time of culture under saline conditions, as a factorial experiment, on carbohydrate concentration of jojoba explants.

Variables	Source of variation		
	Salinity	Time	Salinity x time interaction
Sucrose	<0.01	<0.001	<0.01
Glucose	<0.001	<0.001	>0.05
Fructose	>0.05	<0.001	>0.05
Inositol	>0.05	>0.05	<0.05
Pinitol	<0.01	<0.05	>0.05
Starch	>0.05	>0.05	>0.05
Hexoses	<0.001	<0.001	>0.05
Sucrolysis index	<0.001	<0.001	<0.001
Water content	<0.001	>0.05	>0.05

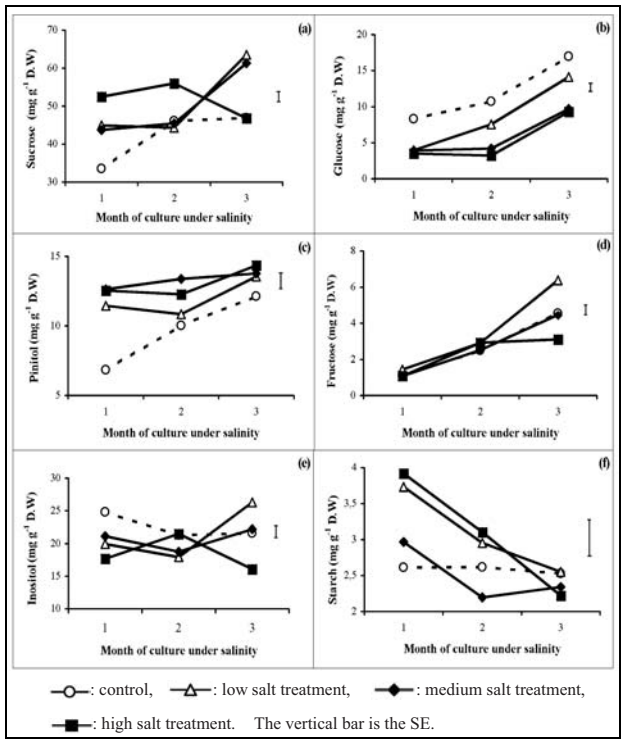


Fig. 1. Fluctuations of jojoba explant soluble sugars (sucrose, glucose, pinitol, fructose, inositol) and starch content under four levels of salinity during the proliferation stage.

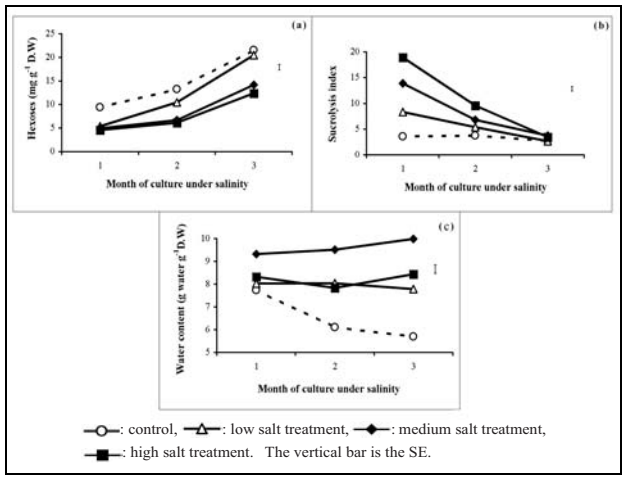


Fig. 2. Fluctuations of jojoba explant carbohydrates, water content and sucrolysis index, under four levels of salinity during the proliferation stage.

Index (estimated as the ratio sucrose/(glucose+fructose)(BALIBREA et al. 2000) was also significantly affected. Most of the variables were affected by the time of culture under saline conditions, except for inositol, water and starch content. Significant interactions between salt level and time of culture under saline conditions were observed only in a few cases.

Most of the time, the sucrose concentration was higher in explants cultured under saline conditions (Fig. 1a). Explants growing in salt free medium had a higher level of glucose during the entire experimentation period (Fig. 1b) than those explants growing in medium supplemented with salt. As the salt concentration increased, glucose concentration decreased, while during the third month there was a significant increase of glucose content in all treatments. Almost the opposite was true for pinitol content, as explants growing under salt free conditions had the lowest content throughout the culture period (Fig. 1c). Its concentration was increasing through time, under almost all salinity levels. Fructose content was increasing with time, while there were significant differences between salt levels only during the third month (Fig. 1d). Inositol concentration in explants cultured under salt conditions exhibited non-consistent changes, as can be seen in Fig. 1e. Starch content of explants decreased with time under all salinity levels (Fig. 1f).

Hexose concentration increased with time, while remaining consistently higher in explants growing under salt free conditions (Fig. 2a). As the salt level increased, the hexose concentration decreased. In contrast, sucrolysis index decreased with time under all salinity levels (Fig. 2b). A higher concentration of salt in the medium resulted in a higher sucrolysis index, especially during the first two months. The water content of explants seemed to be significantly affected by salinity, as explants growing under salt free conditions had the lowest water content, while explants growing at the medium salt level, had the highest water content throughout the experimentation period (Fig. 2c).

Discussion

The two parameters estimated in this experiment, as indices of salt tolerance and growth rate of explants indicated the salt-tolerant nature of jojoba, in agreement with the literature (BENZIONI et al. 1992; MILLS and BENZIONI 1992; BENZIONI et al. 1996; ROUSSOS and PONTIKIS 2003).

Jojoba explants exhibited a significant increase in sucrose content under salinity, as several other plants (SUN et al. 1999; KAFI et al. 2003). Sucrose is a readily translocable carbohydrate (SHEEN et al. 1999) which accumulates in the active growing region and acts as osmoprotectant (PEREZ-ALFOCEA and LARHER 1995), especially under saline conditions (KAFI et al. 2003). The accumulation of sucrose in jojoba explants could possibly be the result of increased uptake from the medium, where sucrose is present in high quantities, rather than the result of *de novo* synthesis. Moreover, sucrose metabolizing enzyme (acid invertase activity) may be inhibited under salinity, resulting in an accumulation of sucrose and reduced glucose content due to low sucrolytic activity (PEREZ-ALFOCEA and LARHER 1995; KAUR et al. 2003).

Glucose on the other hand exhibited a significant decrease with salinity. Significant decreases of glucose content under saline conditions have been reported (PEREZ-ALFOCEA and LARHER 1995) as also decreases of invertase activity resulting in reduced glucose content (KAUR et al. 2003), while in other cases glucose

content increased with salinity (GUCCI et al. 1998; BALIBREA et al. 2000). Reduction of glucose content could be the result of reduced net photosynthesis, due to the reduced stomatal conductivity and the degradation of chlorophyll (KARSTENS et al. 1993; MAKELA et al. 2003; MELONI et al. 2003). Glucose increase with time, could derive either from starch degradation through amylase activity or through sucrose catabolism.

Fructose content was found to be similar in explants growing on salt free medium and on medium supplemented with salt. This carbohydrate was present in small amounts in jojoba explants. Its source could be the small amounts present in the medium after sucrose hydrolysis through autoclaving or may derive from sucrose via acid invertase activity, especially during the third month, in order to maintain explant growth.

Inositol was affected neither by salinity nor by time of culture under salt stress. Its content was probably not affected since inositol was a constituent of the medium. The metabolic pathway of inositol includes its conversion to D-ononitol (through inositol methyl transferase-IMT) and the epimerisation of the latter to D-pinitol (STREETER et al. 2001). Salt stress has been found to increase gene expression of inositol methyl transferase, the key enzyme in the biosynthesis of methylated inositols (SUN et al. 1999). However, D-ononitol was not detected in jojoba explants, in contrast to D-pinitol. This could probably mean that D-ononitol was epimerised rapidly into D-pinitol, which is characterized as an end product, due to its slow metabolism (SUN et al. 1999).

Pinitol was found to be present in higher amounts in explants cultured under saline conditions compared to those deriving from salt free medium. It is well known that polyols and cyclitols can act as osmoprotectants under drought and salt stress, by replacing water at the surface of proteins, protein complexes, or membranes (SUN et al. 1999; NOIRAUD et al. 2001; ZHU 2001), as well as for intracellular osmotic adjustment between the vacuole (high NaCl concentration) and the cytoplasm (low NaCl concentration) (SHEVELEVA et al. 1997; SUN et al. 1999).

On the other hand, starch content was not affected either by salinity or by time. In many experiments amylase activity decreases under salt stress (ASHRAF et al. 2002; KAUR et al. 2003), which means that starch content is either stable or increasing, though its catabolism is reduced. It has been well established, that the partitioning of sugars into starch avoids metabolic alterations and feedback metabolic inhibitions under stress conditions (BALIBREA et al. 2000). Nevertheless, the low amylase activity detected in some studies was enough to maintain a glucose supply to plants, under conditions of reduced growth (ASHRAF et al. 2002). In the present study, starch could contribute to glucose level through low amylase activity, as glucose was increasing with time while in the same time starch was decreasing.

Hexoses and sucrolysis index exhibited almost the opposite pattern of change. It seems that hexose content decreased under salt stress, while the index increased. According to BALIBREA et al. (2000) the high sucrolysis index supports the idea of a blockage in the utilization of carbohydrates in the sink organs. This could possibly result by the high accumulation of sucrose in explants and the low production of glucose and fructose, either due to enzyme inhibition or reduced net photosynthesis. The progressive decrease of the index could indicate the gradual conversion of sucrose into glucose and fructose in order to maintain explant growth.

Water content of explants was high in those cultured under saline conditions. In the present study it seemed that jojoba explants responded to salinity stress by imbibing water, and thus diluting the toxic concentrations of sodium and chloride accumulated (unpubl. data) exhibiting thus the so called mechanism 'salt dilution by succulence' (HAGENMEYER 1996).

The high accumulation of water within jojoba explants is a strong argument for the osmoprotective role of carbohydrates under saline conditions. Accumulation of pinitol and sucrose as a response to excess accumulation of monovalent ions in the vacuole (KAFI et al. 2003) may increase the osmotic potential of the cells, thus maintaining the water potential differences between vacuole and cytoplasm as well as between cells and external medium and avoiding desiccation by improving water absorption. Accumulation of sugars could also serve as plant resources against or upon relief from stress, as has been revealed by the sucrolysis index.

Carbohydrates have a major role in plant adaptation mechanisms against abiotic stress factors. *In vitro* culture could probably offer some new information about their role during salinity stress in early stages of growth and during the very first stages of plant propagation and breeding.

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